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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES**

In re application of: Y.F. Liu

Art Unit: 1631

Serial No.: 09/156,367

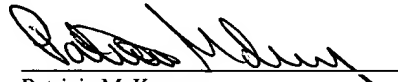
Examiner: Marianne P. Allen

Filing Date: September 17, 1998

For: A METHOD FOR IDENTIFYING MLK INHIBITORS FOR
TREATMENT OF NEUROLOGICAL CONDITIONS

CERTIFICATE OF MAILING UNDER 37 C.F.R. §1.8(a)

The undersigned hereby certifies that this document is being placed in the United States mail with first-class postage attached, addressed to the Commissioner for Patents, Washington, D.C. 20231 on July 16, 2002.


Patricia McKenney

Commissioner for Patents
Washington, DC 20231
ATTENTION: Board of Patent Appeals and Interferences

Sir:

APPELLANT'S BRIEF ON APPEAL

This is an appeal to the Board of Patent Appeals and Interferences from the decision of the Examiner finally rejecting claims 1-3, 5-10, 14-19 and 45, and is in furtherance of the Notice of Appeal filed on February 7, 2002 in this application. The appealed claims are as set forth in the attached Appendix of Claims. Provision for the payment of fees required for filing this brief, and any required extension of time for filing the brief, is submitted herewith. This brief is submitted in triplicate in accordance with the provisions of 37 C.F.R. §1.192(a).

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REAL PARTY IN INTEREST

The real party in interest in this appeal is Dr. Ya Fang Liu, the party named in the caption of this brief.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences that will directly affect, be directly affected by, or have a bearing on the Board's decision in this appeal.

STATUS OF CLAIMS

The status of the claims in this application is as follows. Claims 1-3, 5-10, 12-19 and 45 are pending and are on appeal. Claims 1-3, 5-10, 12-19 and 45 were finally rejected in the Office Action dated November 2, 2000. Claims 4, 11 and 20-44 have been previously cancelled. No claims have been allowed.

STATUS OF AMENDMENTS

An Amendment After Final Rejection was filed on February 7, 2002. The Amendment proposed amending certain claims and canceling other claims. An Advisory Action was mailed on March 21, 2002, in response to the Amendment. In the Advisory Action, the Examiner stated that the Amendment and Response would not be entered since the proposed amendments (i) raised new issues that would require further search and/or consideration, (ii) were not deemed to place the application in better form for appeal, and (iii) with respect to claims 7 and 8, raise new grounds of rejection. A Supplemental Amendment After Final Rejection was filed on April 11, 2002. The Supplemental Amendment proposed certain claim amendments and claim cancellations in response to issues raised in the Advisory Action. The Supplemental Amendment has not been acted on by the Examiner as yet. Appellant believes that entry of the Supplemental Amendment would obviate this appeal.

SUMMARY OF INVENTION

The claimed invention relates to methods for assessing the ability of a compound to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition. Neuronal cells having activated MLK activity are contacted with a compound, and the number of neuronal cells that die is determined. A decrease in the number of dead neuronal cells in the presence of the compound, compared to the number of dead neuronal cells in the absence of the compound, is indicative of the ability of the compound to inhibit neuronal cell death. Page 3, line 23 to page 4, line 2. The neuronal cells can be apoptotic neurons, wherein the cell death is caused by a neurological condition, or neurons that are induced to undergo apoptosis, such as by contacting the neuronal cells with a neurotoxin such as glutamate, quinolinic acid or kainic acid. Page 4, lines 2-5. HN33 hippocampal neuronal cells are preferred. Page 4, lines 5-6.

In other embodiments, the claimed invention also relates to the ability of a compound to inhibit MLK activity by contacting the compound with an MLK, measuring the amount of MLK-associated activity, and comparing the level of the MLK-associated activity in the presence of the compound with the level of MLK-associated activity in the absence of the compound. Page 4, lines 7-16. The MLK-associated activity can be an MLK activity or apoptosis. Page 4, lines 17-19. Apoptosis can be induced in a cell by introducing a huntingtin protein, such as polyglutamine stretch-expanded huntingtin, in the cell, or by introducing the C-terminal 100 amino acids of an amyloid precursor protein (APP). Page 4, lines 20-26.

ISSUES

The issues to be decided in this appeal are as follows:

1. Whether the present claims are entitled to the benefit of the provisional application filing date.
2. Whether claims 1-3, 5-8 and 12-19 are supported by the specification or contain new matter.

3. Whether claims 9, 10 and 45 are enabled by the specification under 35 U.S.C. 112, first paragraph, in such a way as to permit one skilled in the art to make and/or use the invention.
4. Whether claims 2, 7-9 and 12-13 are indefinite under 35 U.S.C. 112, second paragraph, for failing to particularly point out and distinctly claim the invention.
5. Whether claims 1, 6, 14, 19 and 45 are unpatentable under 35 U.S.C. 102(e) as being anticipated by the Miller et al. reference (U.S. Patent No. 6,060,247).
6. Whether claim 19 is unpatentable under 35 U.S.C. 103(a) as obvious over any of the Tibbles et al., Rana et al., and Hirai et al. literature references in view of the Au-Young reference (U.S. Patent No. 5,817,479).

GROUPING OF CLAIMS

Claims 1, 9, 14, 19 and 45 are the pending independent claims in this application. Claims 1 and 9, and claims dependent thereon, are directed to the assessment of compounds of interest by the measurement of neuronal cell death. Claims 2, 3 and 5-8, can be grouped with claim 1, and claims 10, 12 and 13, can be grouped with claim 9. These claims stand or fall together. Claims 15-19, directed to the assessment of compounds of interest by the measurement of the level of apoptosis in neuronal cells, can be grouped with claim 14, and stand or fall with claim 14. Claims 19 and 45, which relate to the assessment of compounds of interest by the measurement of MLK activity, stand or fall together. Claims 1, 2, 3, 5-8, 9, 10, 12 and 13 should be considered independently of claims 14-19 and 45.

ARGUMENT

Benefit of Provisional Application Filing Date

The Examiner has stated that appellant is not entitled to the benefit of the filing date of Provisional Application No. 60/085,439, filed May 14, 1998, since the provisional application does not support the methods as presently claimed, and all of the present claims embrace broader concepts. Appellant respectfully disagrees with this conclusion.

The claims of this application are directed to methods for assessing the ability of a compound for preventing neuronal cell death, and/or for inhibiting MLK activity. For purposes of comparing the provisional and regular applications, the methods of this invention involve the steps of (1) contacting the compound with cultured neuronal cells, or an MLK protein and a substrate for the protein, (2) measuring the response of the cells or protein substrate to the compound, and (3) comparing these results to a control. The issue regarding benefit is whether these methods are disclosed in sufficient detail in the provisional application in order to place one skilled in the art in possession of the claimed invention in compliance with the requirements of the first paragraph of 35 U.S.C. §112. Appellant submits that this is the appropriate standard for benefit purposes, and that appellant has fully complied with this standard.

The provisional application states that polyglutamine-expanded huntingtin causes neuronal toxicity in, for instance, cultured HN33 neuronal cells. As a comparison, 293 normal cells were also evaluated. See pages 7 and 8 of the provisional application, and note that there is no limitation in the independent claims as to the type of compound that can be assessed. Thus, the term "compound", as used in the invention, includes both normal huntingtin and mutated huntingtin within its scope. Moreover, the cells of the invention have activated MLK2 activity as required by the claims. Appellant submits that one skilled in the art, upon reading the provisional application specification, would understand that normal huntingtin has the ability to inhibit neuronal cell death in cells with activated MLK2 activity which are contacted with normal huntingtin, since such cells have been shown to survive. This satisfies the limitations of the independent claims of the present invention. See the relevant disclosure in the provisional application at pages 6-7, and compare Figures 4B and 4C of the provisional application. The provisional application discloses the use of normal huntingtin as only one example of a suitable compound, as one skilled in the art would readily appreciate.

In addition to cell based methods, the provisional application also discloses the use of cell free methods for assessing the effect of a particular compound on neural toxicity. On page 5, the provisional application states that MLK is a protein kinase that directly binds to and activates

SEK1. The expression of mutant huntingtin in MLK-expressing neuronal cells induces cell death. However, when a dominant negative form of SEK1 is co-expressed with mutated huntingtin in MLK-expressing neuronal cells, the death of the cells is blocked. Accordingly, one of ordinary skill in the art would understand that active MLK kinase activity, in binding to and activating SEK1, results in neuronal cell death, and that a compound, such as normal huntingtin, that inhibits MLK2 kinase activity, and the activation of the MLK substrate SEK1, also inhibits neuronal cell death.

Although the present claims may not be supported in *ipsis verbis* by the disclosure in the provisional application, an exact written description of the claims is not a necessary precondition for benefit under 35 U.S.C. 112, first paragraph. Appellant submits that all that is required for benefit is that one skilled in the art be placed in possession of the invention, and appellant has done so here.

Rejections under 35 U.S.C. 112, First Paragraph

Claims 1-3, 5-8 and 12-19 have been rejected under 35 U.S.C. 112, first paragraph, as lacking enablement in view of the fact that the specification does not specifically disclose DLK and LZK activity. The Examiner has stated that this is a new matter rejection, and that the specification as originally filed does not appear to disclose nor contemplate these particular kinases.

Appellant submits that it is not necessary to have literal support in the specification for terms which are disclosed and well known in the prior art. *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). In the present case, the application is directed broadly to both MLK and JNK activity, and states that these terms also include other members of the MLK and JNK family of proteins. See page 8, lines 22-27 of the application. Specific members of the MLK family described in the specification include, for instance, MLK1, MLK2 and MLK3. However, this disclosure of specific examples of MLK activity is not intended to exclude other members of the MLK family which would logically be included within the scope of the invention, and that are recognized in the art as part of this family.

As examples of references that disclose DLK and LZK activities, enclosed with this brief as Exhibit 1 is a literature reference to Sakuma et al., *The Journal of Biological Chemistry*, 272,

pages 28622-28629 (1987). The Sakuma et al. reference describes both LZK and DLK, the relationship of these proteins, and the fact that these proteins are both members of the MLK family of proteins. See, for instance, the abstract on the first page of the reference. In view of this clear disclosure, appellant submits that claims which embrace these two proteins do not create an issue of new matter under prevailing case law.

Claims 9 and 10 have also been rejected under 35 U.S.C. 112, first paragraph, as lacking enablement in the specification. In particular, the Examiner has objected to the expression "occurring in a mammal susceptible to or having a neurological condition". Appellant believe that this expression is fully supported in the specification, and points to page 4, lines 7-9 as evidencing such support. Moreover, appellant believes that one skilled in the art would have no difficulty in determining the precise metes and bounds of this expression as used in the appended claims.

Claim 45 has been rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. According to the Examiner, the specification identifies only kinase enzymatic activity associated with MLK, and that claim 45 is not limited to kinase activity. In addition, the Examiner also contends that there is no requirement in the claim for direct phosphorylation of SEK1 protein, and that the proteins embraced by the claim are large.

Appellant contends that, in fact, claim 45 is no broader in scope than the scope of the enabling disclosure in the specification. Claim 45 specifies that the MLK protein is one of the following activities: MLK1, MLK2 or MLK3. This is certainly consistent with the specification. Moreover, the activity specified in claim 45 is an MLK activity. The MLK activity is further qualified as an enzymatic activity, an ability to bind a SEK1 protein, and an ability to phosphorylate a SEK1 protein. Thus, the proteins embraced by this claim are a clearly defined set of proteins which are required to possess MLK activity in addition to the other listed factors, and are not therefore unduly broad in scope.

Rejections under 35 U.S.C. 112, Second Paragraph

Claims 2, 7-9 and 12-13 have been rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter

applicant regards as the invention. The Examiner states that claims 2 and 9 should have used “and” instead of “or” for proper Markush language. The Examiner also states that claims 8 and 13 are confusing by reciting a “disease comprising”. In addition, the Examiner states that claims 12 and 13 are identical to claims 7 and 8, respectively. Finally, the Examiner states that claims 7-8 and 23-13 are confusing.

Appellant would be prepared to correct the Markush language in claims 2 and 9 should this appeal be successful in all other respects. Moreover, claims 12 and 13 should be dependent on claim 9, rather than claim 1, and appellant would be prepared to correct this oversight as well.

Appellant takes issue with the criticism of the use of the expression “disease comprising”, and believes that the meaning of this expression is clear from the context of the claims. Obviously, the intent of the referenced claim language is to enumerate the specific diseases listed in the claim for which the subject may be susceptible.

Appellant also believes that claim 7-8 and 12-13 are not confusing and further limit the method claims on which they depend. These claims further modify the independent claims on which they depend since they specify the neurological condition of the mammal, and this is an element of the method recited in the independent claims.

Rejections under 35 U.S.C. 102

Claims 1, 6, 14, 19 and 45 have been rejected under 35 U.S.C. 102(e) as being anticipated by Miller et al. (U.S. Patent No. 6,060,247). The Examiner states that the claims recite MLK activity, and that this activity inherently includes the ability to phosphorylate and bind to SEK1, which MEKK1 inherently does. The Examiner also states that the claims do not specifically require kinase activity.

Miller et al. discloses a method for identifying a substance that inhibits apoptosis. According to Miller et al., a population of postmitotic neurons is infected with an adenovirus vector comprising DNA encoding a protein that induces apoptosis. A subset of the cell population is exposed to a substance suspected of inducing apoptosis, and the relative number of apoptotic cells is measured. See, in particular, claim 28 of Miller et al.

The Miller et al. method is distinguished from the methods of present claims 1, 6, 14, 19 and 45 in the following respects. Present claims 1, 6 and 14 are directed to a method for

assessing the ability of a compound to prevent neuronal cell death by contacting the compound with cultured neuronal cells having activated MLK activity. Present claims 19 and 45 involve contacting the compound to be assessed with an MLK protein and a substrate therefore. None of the present claims involve the use of an adenovirus vector to infect the neuronal cells to induce apoptosis, and there is no indication from the reference that the neuronal cells of Miller et al. have increased MLK activity. Accordingly, there is no basis for asserting that Miller et al. anticipates the present claims since the reference does not disclose each and every element of the claims as required

Rejections under 35 U.S.C. 103

Claim 19 has been rejected under 35 U.S.C. 103(a) as being obvious over the Tibbles et al., Rana et al., and Hirai et al. references, each in view of the Au-Young et al. patent (U.S. Patent No. 5,817,479). The Examiner states that the references teach that MLK1, MLK2, and MLK3 directly phosphorylate SEK1, and that in order to do so they must each bind SEK1. The Examiner also states that the ability to phosphorylate is a measure of kinase activity. Appellant respectfully disagrees with this conclusion.

Claim 19 is directed to a method for assessing the ability of a compound to inhibit MLK activity by contacting the compound with an MLK protein and a substrate therefore, measuring the level of MLK activity, and comparing the level of MLK activity in the presence and absence of the compound. The MLK activity is further specified as a kinase activity or an ability to bind an SEK1 protein.

The Tibbles et al. reference discloses that MLK3 catalyzes the phosphorylation of SEK1 *in vitro*. The Rana et al. reference discloses that SPRK and MEK kinase-1 phosphorylate and activate recombinant SEK-1. Finally, the Hirai et al. reference discloses a MAPK kinase class protein, SEK1/MKK4/JNKK, that is activated and phosphorylated by MST/MLK2.

The Au-Young patent describes polynucleotides which encode certain novel protein kinases expressed in human cells and tissues. This patent also states that competitive drug screening assays incorporating neutralizing antibodies capable of binding to kinase fragments can be used to evaluate the binding of test compounds to such kinase fragments. See col. 23, lines 44-50 of the reference.

Other than the fact that all the references relate in some way to kinases, there is no basis for combining these references as proposed by the Examiner. Moreover, the result of such a combination would neither teach nor suggest the method of claim 19 which is directed to the assessment of the ability of a compound to inhibit MLK activity. One skilled in the art would not be properly motivated to develop such a method based on the cited references.

Conclusion

Appellant has demonstrated herein entitlement to the benefit of the filing date of Provisional Application No. 60/085,439, filed May 14, 1998. Moreover, appended claims 1-3, 5-8 and 12-19 are believed to be fully enabled by the specification, and to comply with all other requirements of 35 U.S.C. §112, first paragraph. Claims 2, 7-9 and 12-13 are also believed to be sufficiently definite to overcome any rejections on the basis of 35 U.S.C. 112, second paragraph.

The Miller et al. reference does not anticipate the present claims since there has been no showing on the part of the Examiner that the transfected neuronal cells of Miller et al. possess activated MLK activity. Moreover, the combination of Tibbles et al., Rana et al., and Hirai et al., each in view of the Au-Young et al. patent, does not render the claimed invention obvious since assessing the ability of a compound to inhibit MLK activity is not taught or suggested in any combination of these references.

Accordingly, for the reasons presented in this brief, appellant respectfully urges the Board to reverse the rejections made in the final Office Action, and to allow all of the appended claims.

APPENDIX

1. A method for assessing a compound's ability to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition, comprising:
 - a) contacting a compound with cultured neuronal cells having activated MLK activity, wherein activated MLK activity is selected from the group consisting of MLK1 activity, MLK2 activity, MLK3 activity, DLK activity, LZK activity, and an ability to bind a SEK1 protein; and
 - (b) determining the number of cultured neuronal cells that die;wherein a decreased number of dead cultured cells in the presence of the compound compared to the number of dead cultured neuronal cells in the absence of the compound is indicative of the compound's ability to prevent neuronal cell death.
2. The method of claim 1, wherein the neuronal cells are expressing a mutated protein selected from the group consisting of polyglutamine stretch-expanded huntingtin or C-terminal 100 amino acids of amyloid precursor protein, or treated with a neurotoxin to induce apoptosis.
3. The method of claim 2, wherein the neuronal cells are HN33 cells.
5. The method of claim 2, wherein the neurotoxin is glutamate, quinolinic acid or kainic acid.
6. The method of claim 2, wherein the neuronal cells are apoptotic neurons.
7. The method of claim 1, wherein the neuronal cell death occurs in a mammal having a neurological disease whereby glutamate or kainic acid mediated excitotoxicity is involved in neuronal cell death.

8. The method of claim 1, wherein the neuronal cell death occurs in a mammal having a neurological disease comprising Huntington's disease, Parkinson's disease or Alzheimer's disease.

9. A method for assessing a compound's ability to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition, comprising:

a) contacting a compound with cultured neuronal cells expressing a mutated protein selected from the group consisting of polyglutamine stretch-expanded huntingtin or C-terminal 100 amino acids of amyloid precursor protein, or treated with a neurotoxin to induce neuronal cell death; and

(b) determining the number of cultured neuronal cells that die;
wherein a decreased number of dead cultured neuronal cells in the presence of the compound compared to the number of dead cultured cells in the absence of the compound is indicative of the compound's ability to prevent neuronal cell death.

10. The method of claim 9, wherein the neuronal cells are HN33 cells.

12. The method of claim 1, wherein the neuronal cell death occurs in a mammal having a neurological disease whereby glutamate or kainic acid mediated excitotoxicity is involved in neuronal cell death.

13. The method of claim 1, wherein the neuronal cell death occurs in a mammal having a neurological disease comprising Huntington's disease, Parkinson's disease or Alzheimer's disease.

14. A method for assessing the ability of a compound to prevent neuronal cell death occurring in a mammal susceptible to or having a neurological condition, comprising:

a) contacting a compound with cultured neuronal cells having activated MLK activity, wherein activated MLK activity is selected from the group consisting of MLK1 activity, MLK2 activity, MLK3 activity, DLK activity, LZK activity, and an ability to bind a SEK1 protein;

b) contacting, in the presence of the compound, surviving cells from step (a) with an agent that induces apoptosis; and

(c) comparing the level of apoptosis in the cells in the presence of the compound with the level of apoptosis in the cells in the absence of the compound;

wherein the compound is a potentially useful drug for treating mammals when the level of apoptosis in the cells in the presence of the compound is less than the level of apoptosis in the cells in the absence of the compound.

15. The method of claim 14, wherein the apoptotic agent is a neurotoxin.

16. The method of claim 15, wherein the neurotoxin is glutamate, quinolinic acid, kainic acid.

17. The method of claim 14, wherein step (b) is performed by transfecting the surviving neuronal cells with nucleic acid encoding a mutated form of huntingtin or amyloid precursor protein.

18. The method of claim 14, wherein the neuronal cells are HN33 cells.

19. A method for assessing a compound's ability to inhibit MLK activity, comprising:

a) contacting a compound with a MLK protein and a substrate therefore, wherein the MLK protein is selected from the group consisting of MLK1, MLK2, MLK3, DLK, LZK, and combinations thereof;

b) measuring the level of MLK activity, wherein the MLK activity is selected from the group consisting of kinase activity and an ability to bind a SEK1 protein; and

c) comparing the level of MLK activity in the presence of the compound with the level of MLK activity in the absence of the compound, wherein a decrease in MLK activity in the presence of the compound is indicative that the compound has an ability to inhibit MLK activity.

45: A method for assessing the ability of a compound to inhibit MLK activity and to prevent neuronal cell death, comprising the steps of:

a) contacting a compound with a MLK protein and a substrate therefore, wherein the MLK protein is selected from the group consisting of MLK1, MLK2, MLK3, and combinations thereof;

b) measuring the level of MLK activity, wherein the MLK activity is selected from the group consisting of an enzymatic activity, an ability to bind a SEK1 protein, and an ability to phosphorylate a SEK1 protein;

c) comparing the level of MLK activity in the presence of the compound with the level of MLK activity in the absence of the compound, wherein a decrease in MLK activity in the presence of the compound is indicative that the compound has an ability to inhibit MLK activity;

d) contacting the compound having an ability to inhibit MLK activity with cultured neuronal cells having activated MLK activity, wherein the activated MLK activity is selected from the group consisting of an enzymatic activity, an ability to bind a SEK1 protein, and an ability to phosphorylate a SEK1 protein; and

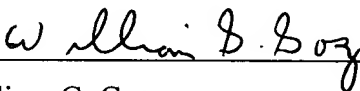
e) comparing the occurrence of apoptosis in the cultured neuronal cells in the presence of the compound with the occurrence of apoptosis in the cultured neuronal cells in the absence of the compound;

wherein the compound having an ability to inhibit MLK activity has the ability to prevent neuronal cell death when the occurrence of apoptosis in the cultured neuronal cells in the presence of the compound is less than the occurrence of apoptosis in the cultured neuronal cells in the absence of the compound.

Appellants hereby authorize the Commissioner, to withdraw the \$320.00 fee for filing this appeal brief, and for the \$1,440 four month extension of time fees, for a total in the amount of **\$1,760.00**, from Appellant's Deposit Account No. 18-1945. If there are any other fees not accounted for above, Appellants hereby authorize the Commissioner to charge the fee to Deposit Account 18-1945.

Respectfully submitted,
ROPES & GRAY

Date: July 16, 2002



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Molecular Cloning and Functional Expression of a cDNA Encoding a New Member of Mixed Lineage Protein Kinase from Human Brain*

(Received for publication, March 4, 1997, and in revised form, August 24, 1997)

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We have cloned a novel protein kinase from human cerebellum and named it LZK (leucine zipper-bearing kinase). The LZK cDNA encoded a 966-amino acid polypeptide that contains a kinase catalytic domain and double leucine/isoleucine zippers separated by a short spacer region. The amino acid sequence of the kinase catalytic domain was a hybrid between those in serine/threonine and tyrosine protein kinases, indicating that LZK belongs to the subfamily of the mixed lineage kinase (MLK) family. The kinase catalytic domain of LZK was most similar to DLK (Holtzman, L. B., Merritt, S.E., and Fan, G. (1994) *J. Biol. Chem.* 269, 30808-30817), MUK (Hirai, S., Izawa, M., Osada, S., Spyrou, G., and Ohno, S. (1996) *Oncogene* 12, 641-650), and ZPK (Reddy, U. R., and Presure, D. (1994) *Biochem. Biophys. Res. Commun.* 202, 613-620), which belong to the same subfamily of the MLK family. However, besides the kinase catalytic domain and double leucine/isoleucine zippers, there was no significant homology with known proteins. The recombinant LZK autophosphorylated in the presence of ATP and divalent cations, and exhibited serine/threonine kinase catalytic activity. Northern blot analysis revealed that LZK is expressed most strongly in the pancreas, with a pattern that differs from other MLKs. Expression of LZK in COS7 cells induced phosphorylation of c-Jun and activation of JNK-1, indicating the association of LZK in the c-Jun amino-terminal kinase/stress-activated protein kinase pathway. The expressed LZK was detected primarily in the membrane fraction, suggesting that LZK interacts with other cellular components *in vivo*.

Protein kinases play critical roles in the regulation of many cellular processes (1), such as the transmission of signals from growth factor (2, 3), control of cell growth and division (4), regulation of cytoskeletal changes (5), gene expression and differentiation (6), translation (7), and metabolism (1). The protein kinases can be divided into two groups based on their sequence similarities and their specificity for the acceptor

amino acid (1, 8, 9). Most protein kinases phosphorylate either serine/threonine or tyrosine, although protein kinases that modify histidine have been found. However, a small number of dual-specificity kinases can phosphorylate both serine/threonine and tyrosine residues (10), although they are structurally related to the serine/threonine-specific group. Protein kinases can also be grouped as receptor protein kinases and non-receptor protein kinases. Receptor protein kinases have an intracellular catalytic domain, transmembrane region, and extracellular ligand-binding domain. Protein kinases share, besides the protein kinase catalytic domain, some structural features reflecting their particular roles in protein-protein interactions. For example, the SH3¹ domains are found not only in tyrosine kinases and serine/threonine kinases but also in receptor-type and non-receptor protein kinases (11, 12). The leucine/isoleucine zipper sequence is found in some protein kinases (13). Recently, many new closely related intracellular kinases have been identified. One of these groups, mixed lineage kinases (MLKs), contains a unique double leucine/isoleucine zipper (14). MLK has a characteristic kinase catalytic domain with a sequence hybrid between those in serine/threonine and tyrosine protein kinases. These kinases include MLK1 (15), MLK2 (16, 17), MLK3/SPRK/PTK1 (18-20), and DLK/ZPK/MUK (21-23). Of these, DLK/ZPK/MUK are considered a secondary subfamily of MLK because of their characteristic sequences. However, little is known about the overall biochemical features and functional roles of MLKs.

We examined the cloning, expression, and preliminary characteristics of the novel intracellular protein kinase. The LZK cDNA encoded a protein with an apparent molecular mass of 135-150 kDa on reducing SDS-PAGE. Sequence analysis revealed that LZK belongs to the MLK family, containing the kinase catalytic domain and leucine/isoleucine zippers. However, LZK had no other strong homologies with any known proteins. The recombinant LZK protein exhibited serine/threonine protein kinase activities *in vitro*. Expression of LZK in COS7 cells induced phosphorylation of c-Jun and activation of JNK-1, suggesting the association of LZK in the JNK/SAPK pathway.

EXPERIMENTAL PROCEDURES

cDNA Library Screening and Sequence Determination of LZK—A 826-bp rat cDNA clone with unknown functions, which had been iso-

* This work was supported in part by the Special Coordination Funds of the Japanese Science and Technology Agency for Promoting Science and Technology and also by a grant-in-aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB001872.

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¹ The abbreviations used are: SH, Src homology domain; MLK, mixed-lineage kinase; MAPKKK, mitogen-activated protein kinase kinase; JNK, c-Jun amino-terminal kinase; SAPK, stress-activated protein kinase; JNKK, JNK kinase; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PVDF, polyvinylidene difluoride; bp, base pair(s); kb, kilobase pair(s); MEKK, MAPK/ERK kinase; LZK, leucine zipper-bearing kinase; MUK, MAPK-upstream kinase; DLK, dual leucine zipper-bearing kinase; ZPK, leucine zipper protein kinase.

lated by screening of the λ ZAP rat brain cDNA library with an antibody raised against a soluble fraction of rat brain, was labeled with [α - 32 P]dCTP by a random primer DNA labeling kit (TaKaRa), and the radiolabeled cDNA was used as a probe to screen approximately 5×10^5 plaques of a human cerebellum cDNA library (CLONTECH). Hybridization was carried out in the buffer consisting of 50% formamide, $5 \times$ SSC, 50 mM phosphate buffer, pH 7.0, 0.5% skim milk, 0.1% SDS, and 100 μ g/ml yeast RNA at 42 °C. Filters were washed at 65 °C in $2 \times$ SSC containing 0.1% SDS and in $0.2 \times$ SSC containing 0.1% SDS sequentially. Three unique clones were isolated and restriction mapped. The longest clone was subcloned into the plasmid vector Bluescript SK, sequenced along both strands over the entire length using a Taq DyeDeoxy terminator cycle sequence kit and an ABI 373A DNA Sequencer (Applied Biosystems).

Analysis of LZK Transcript Expression.—Multiple human tissue Northern blot (CLONTECH) was hybridized to radiolabeled human LZK cDNA fragment (corresponding to nucleotides 1895–3174), which had been amplified by polymerase chain reaction and then labeled with [α - 32 P]dCTP by a random primer method. Hybridization was performed as described above for cDNA screening. The filter was finally washed at 65 °C in $0.1 \times$ SSC and 0.1% SDS, and analyzed by BAS 2000 image analyzer. To ensure the integrity and the quantity of RNA per lane, the blot was rehybridized to radiolabeled β -actin cDNA.

Construction of Epitope-tagged LZK.—The cDNA fragment encoding the LZK open reading frame was engineered with *Xba*I restriction sites, and the product was amplified by long and accurate (LA)-polymerase chain reaction (oligonucleotides: 5'-GCTCTAGAATGGCCAACTTTCAGGAGCACCTGAGCTGCTCTCT-3'; 5'-GCTCTAGATCATTACCAAGGTAGCAGAGCTGTACTGTTTATTGGT-3'). The digested fragment and a double-strand oligonucleotide linker (oligonucleotides: 5'-AGCTTCCACCATGAGAGGATCGCACCACCATCATCACCCT-3'; 5'-CTAGAGTGGTGATGATGGTGGTGGCATTCTCATGGTGA-3') were inserted into the cytomegalovirus promoter-based eukaryotic expression vector pCDM8, which had been double-digested with *Hind*III and *Xba*I. The linker presented above was engineered with *Hind*III (5') and *Xba*I (3') restriction sites, and contained the typical Kozak's consensus sequence and coding sequence for the "MRGSHis₆" epitope (Met-Arg-Gly-Ser-His₆). The MRGSHis₆ tag was inserted into the amino terminus of the LZK coding sequence. The construct was sequenced to confirm Taq polymerase fidelity and maintenance of the appropriate reading frame.

Expression of the LZK Constructs.—COS7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and kanamycin. Cells (2×10^6) plated onto a 10-cm tissue culture dish were grown overnight and transiently transfected with 10 μ g of the eukaryotic expression plasmid using LipofectAMINE™ (Life Technologies, Inc.) according to the manufacturer's protocol. After 48 h, cells were washed twice in ice-cold phosphate-buffered saline and then lysed by adding 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 0.2% Triton X-100, and protease inhibitors). The lysate was sonicated on ice and then centrifuged for 20 min at 105,000 $\times g$ at 4 °C.

For immunoprecipitation, 2 μ g of anti-MRGSHis₆ antibody (QIAGEN) and 40 μ l of anti-mouse IgG-Sepharose (Sigma) (50% v/v) were added to the supernatant of the cell lysate, and the mixture was incubated at 4 °C overnight. Beads were washed five times in HNTG buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). For cell fractionation study, the cells were homogenized in the lysis buffer without Triton X-100, and the homogenate was centrifuged as above.

For immunoblot analysis, 20 μ l of cell lysate or the immunoprecipitates were separated under reducing conditions on a 7% SDS-polyacrylamide gel according to Laemmli (24). Proteins were electrically transferred onto nitrocellulose membranes, blocked for 2 h in Tris-buffered saline (TBS, pH 7.5) containing 3% nonfat dry milk, followed by incubation with the MRGSHis₆ antibody or the rabbit anti-LZK immune serum diluted 1:2000 in TBS containing 3% nonfat dry milk and 0.05% Tween 20, and then probed with appropriate horseradish peroxidase-conjugated second antibodies. Blots were developed using the chemiluminescent reagent (Pierce) and subjected to autoradiography.

Detection of Kinase Activity In Vitro.—Immunoprecipitated MRGSHis₆-LZK protein was washed four times with kinase assay buffer (25 mM Hepes, pH 7.2, 10% glycerol, 100 mM NaCl, 10 mM MgCl₂, 5 mM MnCl₂, 0.1 mM sodium orthovanadate, and protease inhibitors). Immunoprecipitates were incubated in 50 μ l of kinase buffer containing 30 μ M ATP and 50 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol, Amersham) for 30 min at 30 °C, then resolved by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto PVDF membrane and then analyzed using

a BAS 2000 image analyzer.

Phosphoamino Acid Analysis.—Phosphoamino acid analysis was carried out as described by Zheng and Guan (25). Following the *in vitro* kinase assay, the radioactive band of 135–150 kDa was excised from the PVDF membrane. The strip was incubated in 1 ml of 6 M HCl at 105 °C for 2 h. After removing of the strip, the sample was dried in a SpeedVac, then washed and dried twice in 1 ml of H₂O. The resulting amino acids were separated on a cellulose plate by one-dimensional electrophoresis. Phosphoamino acid standards were visualized by ninhydrin staining, and radioactivity was detected by a BAS 2000 image analyzer.

Immunoblot Analysis of c-Jun.—Cells (2×10^6) were transiently transfected with the expression vector harboring epitope-tagged LZK. After 48 h, cells were washed with ice-cold phosphate-buffered saline three times and then lysed *in situ* with 1 ml of Laemmli sample buffer. For control experiment, cells were stimulated by UV radiation (100 J/m²). After 1 h, they were used for the experiment. Cell lysate (20 μ l) was subjected to SDS-PAGE, and separated proteins were transferred on to the nitrocellulose membrane. The membrane was blocked by soaking in TBS containing 3% nonfat dry milk, incubated with diluted anti-c-Jun antibodies (0.1 μ g/ml in TBS containing 3% nonfat dry milk and 0.05% Tween 20), and subsequently with appropriately diluted horseradish peroxidase-conjugated secondary antibodies. The resulting membrane was developed using the chemiluminescent reagent and subjected to autoradiography.

Detection of the JNK1 Activities.—Cells (2×10^6) were transiently transfected with the expression vector harboring epitope-tagged LZK. After 48 h, cells were washed three times with ice-cold phosphate-buffered saline and lysed with 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 20 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin, and 2 μ g/ml pepstatin). After removal of insoluble materials by ultracentrifugation, 2 μ g of anti-JNK1 antibody (Santa Cruz Biotechnology) and 200 μ l of protein G-Sepharose (Sigma) (10% v/v) were added to the supernatant of the cell lysate, and the mixture was incubated at 4 °C overnight. Beads were washed four times in buffer consisting of 50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 0.1% Triton X-100, 1 mM sodium orthovanadate, 50 mM NaF, 20 mM β -glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 2 μ g/ml aprotinin and 2 μ g/ml pepstatin, and subsequently washed four times in buffer consisting of 20 mM Hepes, pH 7.5, 15 mM MgCl₂, 15 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, and 2 mM DTT. The immunoprecipitates were incubated for 30 min at 30 °C in 30 μ l of the same buffer containing 25 μ M ATP, 10 μ Ci of [γ - 32 P]ATP (3000 Ci/mmol, Amersham), and 2.5 μ g of glutathione S-transferase-c-Jun. Reactions were terminated by addition of the Laemmli sample buffer. The samples were boiled, resolved by SDS-PAGE, and then analyzed by using a BAS 2000 image analyzer.

RESULTS

Isolation of a LZK cDNA and Its Deduced Amino Acid Sequence.—A 826-bp rat cDNA fragment with unknown function was used as a probe to screen a human cerebellum cDNA library. Three independent clones were isolated, and their inserts were sequenced. The nucleotide sequence of the longest insert is shown in Fig. 1. The cDNA extends over 3450 nucleotide bases and contains 272 bp of 5'-untranslated nucleotides, a continuous open reading frame of 2898 bp, and 399 bp of 3'-untranslated nucleotides. The putative initiation codon was assigned at nucleotide 273. This methionine codon is located within a sequence context favorable for the Kozak's rule and is preceded by an in-frame stop codon beginning at base 234. Within the 3'-untranslated region, putative polyadenylation signals are found at 3318 bp (AATAA), at 3354 bp (AATAA), at 3370 bp (AATTAA), and at 3517 bp (AATAA) upstream from the poly(A) tract. The longest open reading frame of the cDNA encodes a putative polypeptide of 966 amino acids, with a calculated molecular mass of 108 kDa. Hydrophobicity analysis revealed that the protein contains no obvious signal sequence or transmembrane domain (data not shown). Comparison of the sequence with other known proteins revealed that the protein can be divided into several structural domains: a kinase catalytic domain, a double leucine/isoleucine zipper separated by a short spacer region, and an acidic domain at its carboxyl-

FIG. 1. The nucleotide and deduced amino acid sequences of human LZK cDNA. Nucleotide and amino acid numbers are indicated at the left-hand side of each lane. An asterisk (*) denotes the position of the in-frame stop codon located upstream of putative initiation methionine. Putative locations for the polyadenylation signal sequence are underlined. Arrows denote boundaries of the kinase catalytic domain. Hydrophobic residues occupying the *d* positions in putative leucine/isoleucine zipper domains are circled. The completely conserved amino acid sequence (SSEEEGEVDSEVE) commonly found in carboxyl-terminal domain of LZK and ZPK/DLK/MUK is boxed.

Following the kinase catalytic domain, LZK contained two

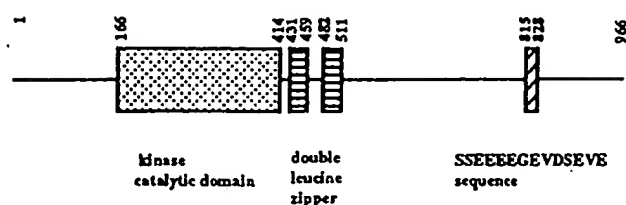


Fig. 2. Schematic representation of human LZK primary structure. The numbers above the diagram represent amino acid residue number and delineate boundaries of indicated domains (motifs). The kinase domain (stippled box), leucine/isoleucine zipper domains (striped box), and the SSEE EGEVDSEVE sequence (slash) are shown. Between two leucine/isoleucine zippers, there is a spacer region.

heptad repeats of nonaromatic hydrophobic amino acids separated by a 25-amino acid spacer. By Chou and Fasman analysis (32), this amino acid sequence formed an α -helix structure, indicating that these regions of LZK are composed of two leucine/isoleucine zipper motifs (Figs. 2 and 3), which may promote homo- or heterodimerization of proteins through hydrophobic interactions. As shown in Fig. 4, hydrophobic residues are conserved at the *d* position in zipper 1 and 2, forming a hydrophobic stripe on the face of the helix. Except for the *d* position, these regions are comparatively rich in charged amino acids. In particular, position *b* (EETE) and position *f* (KSRR) in zipper 1, and position *g* (IRRK) in position 2 were primarily composed of negatively or positively charged amino acids, suggesting that they are involved in intra- or intermolecular electrostatic interactions (33, 34).

The regions containing the kinase catalytic domain and leucine zipper domain of this protein have 86.4% and 86.4% identity, respectively, to previously reported proteins DLK (dual leucine zipper-bearing kinase) (21) and ZPK (leucine zipper protein kinase) (22) (see Fig. 3). In addition, the sequence of this region was homologous to MLK1 (15), MLK2 (16, 17), and MLK3 (18–20) by 40.2%, 40.4%, and 39.5%, respectively (Figs. 3 and 5), suggesting that LZK, together with DLK/ZPK, belongs to the MLK (mixed lineage kinase) family, although no strong similarity was found outside this region. However, in contrast to the other of MLKs, which have a SH3 domain at their amino-terminal ends, LZK (as well as DLK/ZPK) did not contain such a structure (Fig. 5). In addition, LZK and DLK/ZPK have a single invariant Glu at 7 amino acid residues downstream from the invariant Lys in subdomain II, but this is not the case with ordinary MLKs. This Glu residue is believed to play an important role in stabilizing ATP in the ATP-binding site from the crystallographic study (35). These results suggest that LZK, together with DLK and ZPK, belongs to the secondary subgroup of MLK. In addition, LZK and DLK/ZPK share a unique sequence, Ser-Ser-Glu-Glu-Glu-Glu-Gly-Glu-Val-Asp-Ser-Glu-Val-Glu (Ser⁸¹⁵-Glu⁸²⁸ in LZK) (Figs. 5 and 6). However, the glycine/proline-rich region present in DLK/ZPK at the carboxyl- and amino-terminal ends was not detected in LZK (Fig. 5). It should be noted that the sequence of the LZK kinase catalytic domain is 94.6% identical with that of a partial putative serine/threonine protein kinase (36), implying that these proteins are identical or closely related (Fig. 3).

Tissue Distribution of LZK mRNA—Expression of LZK mRNA was examined by Northern blotting mRNA from several human tissues. The probe used for this analysis was corresponded to nucleotides 1895–3174 (See Fig. 1). Three bands at about 9.5, 8.7, and 6.5 kb were found with pancreas mRNA at the highest level. These bands were also markedly detected in the brain, liver, and placenta, and no positive signal was detected in the heart, lung, skeletal muscle or kidney (Fig. 7A). The expression levels of these three transcripts varied among the tissues. The 8.7-kb band was detected only in mRNA from

pancreas. Similarly, the >9.5-kb band was detected only with pancreas and brain. After initial probing with LZK cDNA, the blot was rehybridized with β -actin cDNA to confirm the integrity of the RNA from different tissues (Fig. 7B).

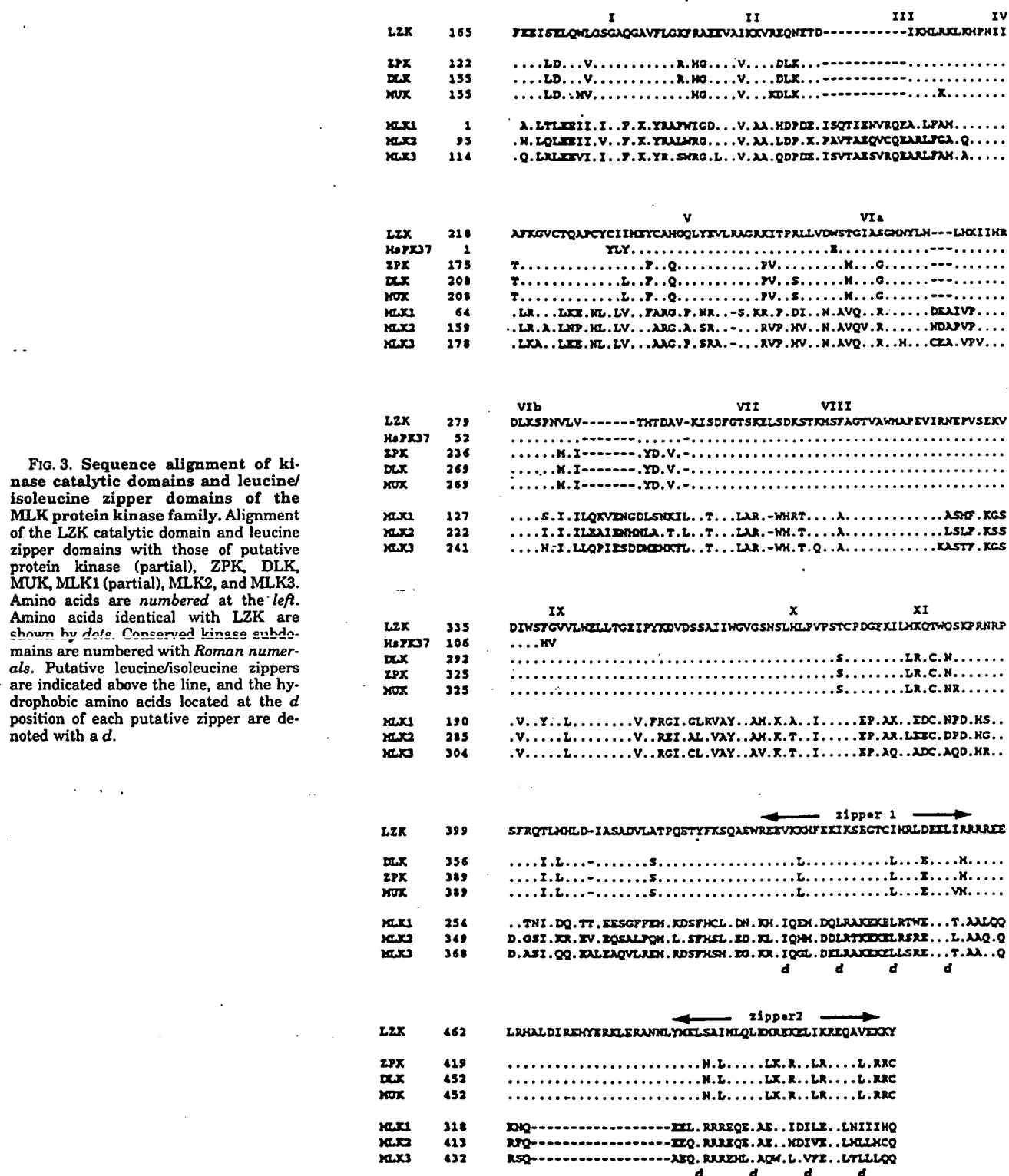
Expression of LZK cDNA in COS 7 Cells and in Vitro Phosphorylation of the Recombinant LZK—To facilitate the detection and immunoprecipitation of the LZK, MRGSHis₆ epitope was incorporated at the amino terminus of LZK (see "Experimental Procedures"). The epitope-tagged full-length LZK cDNA was incorporated into the eukaryotic expression vector pCDM8, and the resulting plasmid was transfected into COS 7 cells. Upon immunoblot analysis of LZK transfectants following the SDS-PAGE under reducing conditions, a protein with a molecular mass of 135–150 kDa, which is in good agreement with the predicted mass of the epitope-tagged LZK, was detected, while no band was detected for the non-transfectant (Fig. 8A). In addition, a protein of 135–150 kDa was specifically immunoprecipitated with a MRGSHis₆ antibody from the lysate of the transfectant (data not shown).

To study the subcellular localization of LZK, COS7 cells expressing LZK were homogenized in the absence of detergent. The homogenate was fractionated into the soluble and the membrane fractions, and the respective fractions were subjected to SDS-PAGE and followed by immunoblot analysis. Strong immunoreactive bands were detected in the membrane fraction, while only weak bands were found in the soluble fraction (Fig. 8B), suggesting that LZK protein binds to some membrane components probably through interaction with some other cellular components such as lipid and/or anchor protein.

To confirm that LZK is an active protein kinase, MRGSHis₆ antibody immunoprecipitates of the LZK transfectants were incubated with [γ -³²P]ATP in the presence of Mn²⁺, Mg²⁺, and Na₃VO₄ (protein-tyrosine phosphatase inhibitor), and then the proteins were separated by SDS-PAGE under reducing conditions followed by transfer onto PVDF membranes. Upon autoradiography, immunoprecipitates from the transfectants revealed radioactive bands of 135–150 and 50 kDa, but no detectable bands in non-transfectants (Fig. 9B). The radioactive band of 50 kDa comigrated with the band of heavy chain of IgG, indicating that LZK not only autophosphorylated itself but also phosphorylated heavy chain of IgG.

The radioactive 135–150-kDa band of LZK from the *in vitro* kinase assay was excised and subjected to partial acid hydrolysis. The resulting materials were separated by one-dimensional electrophoresis on a cellulose plate (25). Analysis by autoradiography and comparison to ninhydrin-stained phosphoamino acid standards revealed only phosphoserine and phosphothreonine (Fig. 9C), indicating that LZK has a serine/threonine kinase activity. However, the present experiment cannot completely exclude the possibility that LZK has a tyrosine kinase activity.

Activation of JNK Pathway by LZK—Recent studies show that some MLKs activate JNK pathway (23, 26, 27). JNK pathway is believed to be predominantly activated by cellular stresses such as UV radiation, inflammatory cytokines, and osmotic shock (28, 29), which results in the activation of transcriptional factors such as c-Jun and ATF2 (30, 31). Because the amino acid sequence of LZK showed high homology to DLK/MUK, which were known to activate JNK pathway, we tested whether or not LZK activates the phosphorylation of c-Jun. COS7 cells were transiently transfected with the expression vector harboring an epitope-tagged LZK, after which the mobility delay of endogenous c-Jun was monitored by immunoblot analysis with anti-c-Jun antibodies. As shown in Fig. 10, expression of LZK induced the mobility delay of c-Jun as much



as was observed with UV radiation. Because the mobility delay is caused by the phosphorylation of c-Jun, these results suggested that expressed LZK activates the endogenous JNK pathway (28). Then to confirm that the phosphorylation of c-Jun observed was really caused by activation of JNK, endogenous JNK1 was immunoprecipitated from the cell lysate and JNK1 activity was determined by *in vitro* kinase assay using soluble glutathione *S*-transferase-c-Jun as substrate. As shown in Fig. 11, expression of LZK elevated the JNK1 kinase activity. The

extent of JNK1 activation by expression of LZK was comparable to that caused by UV radiation. These results taken together indicated that LZK can effectively activate JNK pathway.

DISCUSSION

We examined the cDNA cloning, expression, and characteristics of a novel protein kinase, which is expressed in a spatially regulated fashion in adult human tissues. This protein kinase

FIG. 4. Helical wheel representation of the leucine/isoleucine zippers of LZK. The residues of putative leucine/isoleucine zippers of LZK were arrayed on a helical wheel. The spokes of the wheel show the relative positions of the amino acids in an α -helix, and the positions a - d correspond to the location of the amino acid residues. In an ideal α -helix, amino acid residues appear on one side of the helix in every two turns. In this model, conserved hydrophobic amino acids were located at the d position.

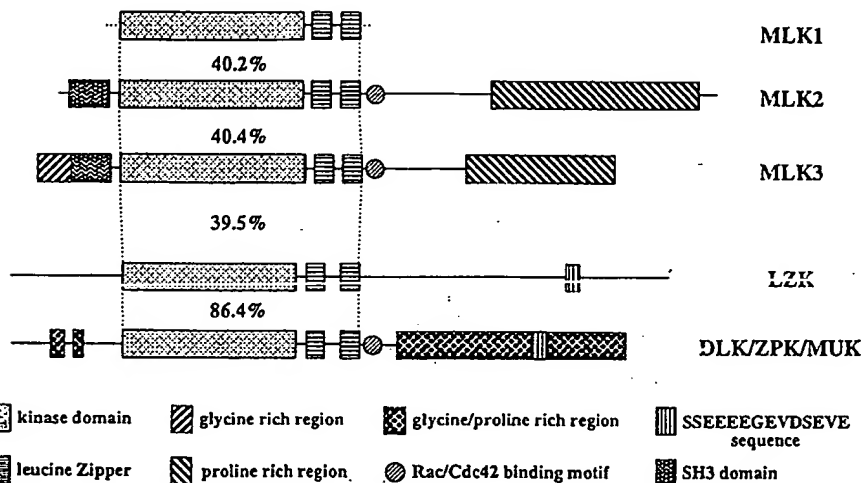
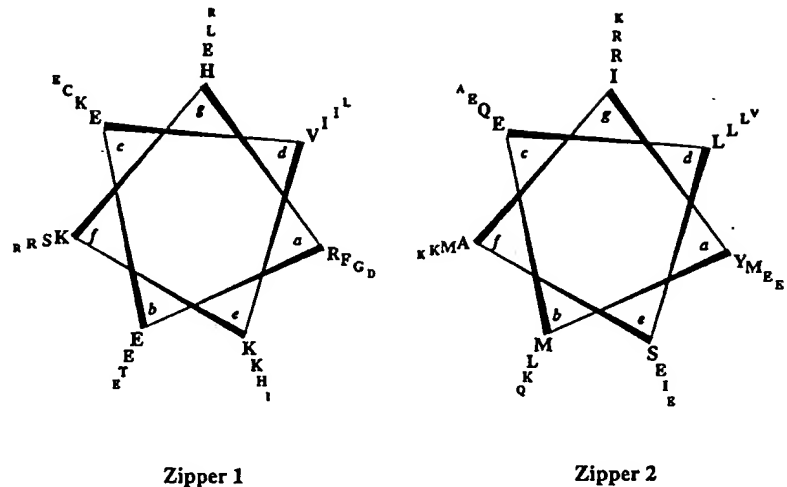


FIG. 5. Schematic representation of the structures of LZK and other MLKs. SH3 domains, glycine-rich regions, proline-rich regions, glycine/proline-rich regions, kinase domains, leucine zippers, Rac/Cdc42 binding motifs, and SSEEVEGEVDSEVE sequences are shown.

H-LZK	805	TRPLQKSGDDSSSEEVEGEVDSEVEFPRQRPHRCISS
ZPK (human)	718	AVTRSQKRGISSEEVEGEVDSEVELTSSQRWPGSLNM
DLK (mouse)	735	AVTRSQKRGISSEEVEGEVDSEVELPPSQRWPGQPNM
MUK (rat)	741	AVTRSQKRGISSEEVEGEVDSEVELPPSQRWPGQPNM

FIG. 6. The amino acid sequence SSEEVEGEVDSEVE were commonly conserved in the carboxyl-terminal domain of LZK and ZPK/DLK/MUK. Besides the regions of kinase catalytic domain and leucine/isoleucine zippers, LZK does not show strong similarity with other protein kinases of the MLK family (see the text). However, the short amino acid sequence SSEEVEGEVDSEVE is completely conserved in the carboxyl-terminal domain of LZK and DLK/ZPK. The amino acid numbers are indicated on the left side of the sequences.

contains a kinase catalytic domain, followed by two leucine/isoleucine zipper motifs, which are separated by a short spacer region. We designated this protein kinase as LZK. The LZK cDNA encodes a protein with an apparent molecular mass of 135–150 kDa, and has serine/threonine kinase activity.

LZK is most similar to DLK and ZPK. DLK was identified by Holzman (21) as a novel protein kinase with a unique kinase catalytic domain, the expression of which is regulated spatially and developmentally. ZPK is cloned and identified as a novel putative protein kinase, which is up-regulated in retinoic acid-treated NT2 cells (22). When the region containing the kinase catalytic domain and the leucine/isoleucine zipper domain of LZK was aligned to DLK and ZPK, homology was 86.4% and 86.4%, respectively, with no insertion and/or deletion. Like

DLK and ZPK, LZK had invariant Glu at the specific location 7 amino acids downstream from invariant Lys in subdomain II. From crystallographic study and structure-function analysis of other protein kinases, the invariant Glu in subdomain III and invariant Lys in subdomain II are believed to play an important role in stabilizing ATP in the ATP-binding site.

The amino acid sequence WMAPE in subdomain VIII is often found in Raf family protein kinases, suggesting that LZK has a MAPKKK-related activity. It is interesting to note that Hirai *et al.* (23) recently identified MUK, which corresponds to rat homologue of DLK (mouse) and/or ZPK (human), as a MAPKKK-related protein kinase such as c-Raf and MAPK/ERK kinase kinase (MEKK) (37). They showed that MUK phosphorylates and activates JNKK *in vivo* and *in vitro*. JNKK (38, 39) can be phosphorylated and activated by the MAPKKK-related kinase, MEKK (40, 41), and acts on Jun kinases, resulting in activation of c-Jun (29, 42). MUK-transfected cells induced hyperphosphorylation of c-Jun, suggesting that MUK can regulate the JNK/SAPK pathway *in vivo*. The induction of JNK was also observed in a truncated MUK consisting of the kinase catalytic domain and leucine/isoleucine zipper motifs, the amino acid sequence of which was 86.4% identical to that of LZK. As might be expected from this high homology with MUK, LZK was in fact shown to induce phosphorylation of c-Jun and activation of JNK1, indicating that LZK stimulates the JNK/SAPK pathway. The extent of JNK1 activation by LZK expression was comparable to that caused by UV radiation. Consid-

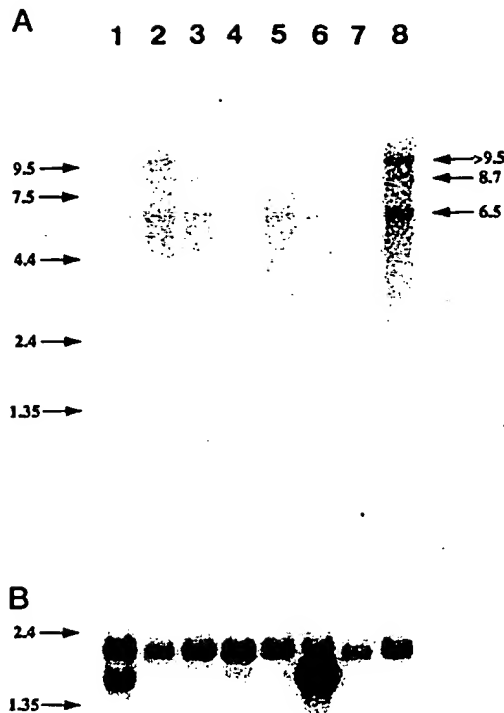


FIG. 7. Expression of LZK mRNA in adult human tissues. The Northern blot was purchased from CLONTECH. In each lane, 2 μ g of poly(A)⁺ RNA from human tissues were loaded. *A*, the blot was hybridized to a radiolabeled probe corresponding to LZK nucleotides 1895–3174. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. *B*, the blot was rehybridized with a radiolabeled actin probe to confirm the integrity of the RNA.

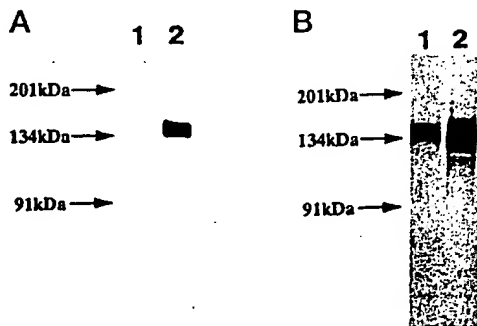


FIG. 8. Expression and cellular localization of epitope-tagged LZK in COS7 cells. *A*, the MRGSHis₆-tagged LZK was transiently transfected into COS7 cells. Transfectants and non-transfectants were lysed in the presence of detergent (see "Experimental Procedures"), and the lysate was resolved by SDS-PAGE under reducing conditions, followed by immunoblot analysis with a MRGSHis₆ antibody. Lane 1, non-transfectant; lane 2, transfectant. *B*, COS7 cells transiently transfected with epitope-tagged LZK were lysed in detergent-free lysis buffer (see "Experimental Procedures") and fractionated by ultracentrifugation. The resulting supernatant (soluble fraction) and pellet (insoluble fraction) were resolved by SDS-PAGE under the reducing conditions, followed by immunoblot analysis with MRGSHis₆ antibody. The loaded materials in each lane corresponded to the equal numbers of the cells. Lane 1, soluble fraction; lane 2, insoluble fraction.

ering the efficiency and cytotoxicity of the transfection procedure, it seems reasonable to speculate that LZK directly phosphorylates and activates the main components of JNK pathway, such as JNKK and MEKK *in vivo*.

When expressed in COS7, LZK was present in both cytosol and membrane fractions. Because LZK contains no obvious

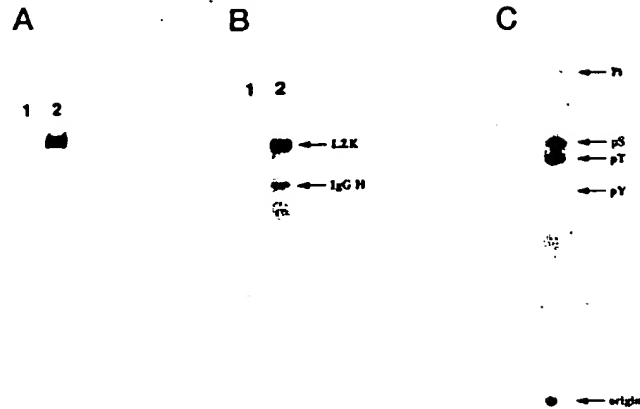


FIG. 9. Immunoprecipitation, autophosphorylation, and phosphoamino acid analysis of LZK. *A*, expressed epitope-tagged LZK was immunoprecipitated from cell lysate as described under "Experimental Procedures." Immunoprecipitates were subjected to SDS-PAGE, followed by immunoblot analysis with MRGSHis₆ antibody. Lane 1, non-transfectant; lane 2, transfectant. *B*, immunoprecipitates were incubated in kinase assay buffer containing [γ -³²P]ATP and divalent cations, subjected to SDS-PAGE, transferred onto the PVDF membrane, and analyzed by BAS 2000. Lane 1, immunoprecipitates from non-transfectant; lane 2, immunoprecipitates from LZK-transfected cells. *C*, radioactive band of 130–140 kDa that corresponds to autophosphorylated LZK was excised and subjected to partial acid hydrolysis. Resultant material was resolved by one-dimensional electrophoresis. pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; Pi, free phosphate.

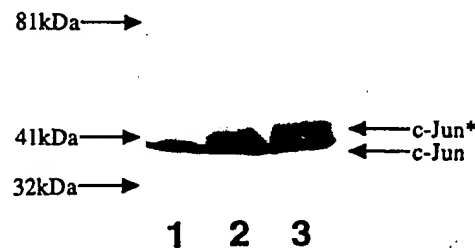


FIG. 10. Hyperphosphorylation of c-Jun by LZK. Cells were lysed with the Laemmli sample buffer and then subjected to SDS-PAGE under reducing conditions, followed by immunoblot analysis with anti-c-Jun antibodies. Lane 1, control cells (non-transfectants); lane 2, cells stimulated by UV radiation (100 J/m²); lane 3, cells transfected with pCDM8-LZK.

signal sequence or transmembrane domain, LZK should first be synthesized in cytosol and then translocated to membranes. It has been thought that subcellular compartmentalization is crucial in providing specificity in the regulation and function of protein kinases (43). Some protein kinases were targeted in a given compartment in the cell, and following various stimulations, they translocated to new sites within the cell, where they associated with anchor proteins, regulated by other protein and/or lipid, to gain access to their physiological substrates. Mata *et al.* (44) recently reported that DLK also exists in both cytosolic and membrane-bound form. They showed that each form of DLK has different biochemical characteristics. The membrane-bound form of DLK is not phosphorylated and forms high molecular complexes, and the cytosolic form of DLK is phosphorylated and exists as monomers. Since LZK, unlike other related protein kinases, does not contain a SH3 domain or a proline-rich region that is a presumed SH3-binding motif, it remains to be clarified what kind of interaction induces the translocation of LZK and regulates the functions of LZK. Recently, MLK-3 was shown to interact specifically with the GTP-bound form of Rac and Cdc42 (45), which regulates the JNK signaling pathway leading to the c-Jun post-transcriptional

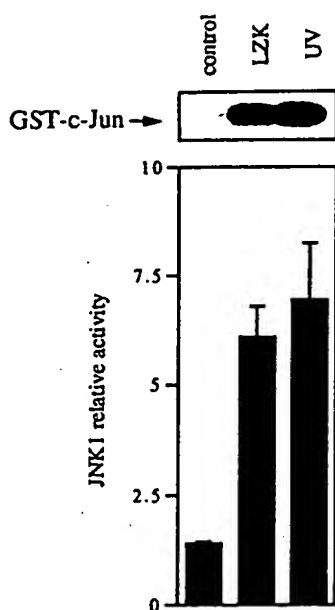


FIG. 11. Activation of JNK-1 by LZK. Endogenous JNK1 was immunoprecipitated from COS7 cells and incubated with glutathione S-transferase-c-Jun in the presence of [γ - 32 P]ATP (see "Experimental Procedures"). The reaction was stopped by the addition of the Laemmli sample buffer, and the samples were separated by SDS-PAGE and then analyzed by using the BAS 2000 image analyzer. The data shown in the upper panel were quantified and are shown in the graph. Data represent the mean \pm S.E. of three independent experiments and are expressed as JNK1 relative activity (lower panel).

activation (46–48). Considering that structurally related MUK activated the JNK pathway, LZK might be associated with mitogen-activated protein kinase pathways under the regulation of a small GTP-binding protein. However, to clarify the mechanism which might regulate the function of LZK, further studies must be done on the biochemical difference between phosphorylated and non-phosphorylated forms of LZK and the signals regulating the compartmentalization of LZK.

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